Neurochemical Evidence of Heterogeneity of Presynaptic and Somatodendritic Nicotinic Acetylcholine Receptors[a]

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Since Langley[1] first reported the existence of a "receptive substance" for nicotine in skeletal muscle that could mediate the effects of nicotine, evidence was obtained that acetylcholine receptors (AChRs) could be involved in synaptic transmission in many parts of the central nervous system (CNS), and also could be responsible for signal transmission through autonomic ganglia. Langley[1] wrote, "The stimuli passing the nerve can only affect the contractile molecule by the radical which combines with nicotine and curare. And this seems in its turn to require that the nervous impulse should not pass from nerve to muscle by an electrical discharge, but by the secretion of a special substance at the end of the nerve." In the paper in which he discussed the data obtained in striated muscle, he was talking about a "contractile molecule" and "radical" that combines with nicotine and curare; he rejected electrical transmission and suggested chemical transmission by a "special substance" secreted from the nerve terminals. He was a visionary.[1,2] Since then enormous progress has been made, and the endogenous special substance was identified as acetylcholine (ACh), and its receptors were shown to be located post- and presynaptically. Sir Henry Dale[2] was the first to suggest in a paper published in 1914 that ACh exerts its action via two different receptors, via muscarinic and nicotinic receptors. At first it was believed that the nicotinic action of ACh is limited to the neuromuscular junction and that its muscarinic action is manifested only at intestinal parasympathetic effector sites. Later it became evident that ACh also stimulates preganglionic nicotinic receptors of the autonomic nervous system, presynaptic nicotinic AChRs (nAChRs) located on the motor nerve terminal, cholinergic receptors located on axon terminals in the CNS and the autonomic nervous system, and cholinergic effector cells in various organs (e.g., head, intestines). ACh, like a master key, fits and stimulates all such muscarinic and nicotinic receptors. Therefore, the effect of ACh released from cholinergic axon terminals depends on the localization of these ACh-sensitive

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receptors. It was recently shown that nicotinic receptors on neurons represent a family of receptors distinct from the well-characterized AChRs of skeletal muscle.[3,4] This area of research has received significant attention because of studies with receptor-ligand binding and molecular biological cloning techniques showing that there are subtypes of nAChRs: receptors located on the muscle are different from those present in the brain. The disadvantage of the receptor binding technique is that it cannot be used for precise localization of the receptors because the ligands used are not selective. It does give an overall characterization of nicotinic receptors of different morphological localization; however, neither of these two methods could differentiate between the receptors in their different neuronal localization. Nevertheless, functional diversity of neuronal nAChRs was shown.[5]

AChR has become one of the best-characterized ligand-gated ion channels with respect of structure, kinetics, pharmacology, and molecular composition. Nicotinic receptors have been postulated to be involved mostly in postsynaptic signaling responses. At present we know much more about the binding properties and

TABLE 1. Neurochemical Evidence That Stimulation of nAChRs Results in Transmitter Release in the Central Nervous System

Transmitter	nAChR Agonist	Brain Region	Reference
NE	DMPP	Human neuroblastoma	63
	Nic	Hippocampus	8
DA	Nic	Striatal synaptosome	11, 12
	Nic	Nucleus accumbens	64
	Nic	Mouse striatal slice	16, 17
	Nic	Rat striatal slice	65
	Nic	Rat striatum	13, 66
ACh	Nic	Cerebral synaptosome	7
	MCC	Rat brain	67
	MCC	Cerebellar synaptosome	33, 68
	Nic	Cerebral cortex slice	69
GABA	Nic	Hippocampal synaptosome	70
	Nic	Cerebral cortex slice	69

Abbreviations: NE, norepinephrine; DMPP, dimethylphenyl piperazinium iodide; Nic, nicotinic; DA, dopamine; ACh, acetylcholine; MCC, methylcarbamylcholine; GABA, γ-aminobutyric acid.

structure of neuronal nAChRs than other aspects. However, a full understanding of the process of chemical neurotransmission in both the peripheral and central nervous system must take into account the role of modulatory presynaptic/prejunctional nicotinic receptors. Neurochemical evidence has been provided that stimulation of nAChRs located on the axon terminals results in release of different transmitters. In the CNS it was found (TABLE 1) that nAChR stimulation enhances the release of ACh from the cortex[6,7] and that of norepinephrine (NE)[8] and serotonin[9] from the hippocampus, and that it increases the resting release of dopamine (DA) from both striatal synaptosomal[10–12] and slice preparations.[13–17] In the peripheral nervous system, nicotinic receptor stimulation facilitates the electrical stimulation-evoked release of ACh from the phrenic nerve terminals in the diaphragm[18] and the release of NE from the pulmonary artery[19] and vas deferens.[20] Several subtypes of nAChRs have been distinguished by pharmacological methods[4,20–24] and molecular biological techniques.[25–27]

In this report, we describe studies of the effects of different nAChR agonists on the release of different transmitters and the modulatory role of presynaptic nicotinic receptors in chemical signal transmission. In addition, using different nAChR agonists and antagonists we attempted to characterize the presynaptic nAChRs located on the noradrenergic axon terminals in the hippocampus and vas deferens and the somatodendritic nAChRs located on the myenteric plexus. The advantage of release studies is that one can be certain where the transmitter comes from, and thus can locate the site of action of different nAChR agonists and antagonists, making the characterization of the receptors rather specific.

RESULTS AND DISCUSSION

Functional Role of Presynaptic nAChRs in Chemical Transmission

The functional role of nicotinic postsynaptic/postjunctional cholinoceptors in chemical neurotransmission is clearly established: in fact, these are the most thoroughly studied of all receptors. However, to understand the effect of stimulation by ACh, the endogenous ligand of nAChRs, on the process of chemical neurotransmission and the mechanism of action of nicotine in both the peripheral and central nervous system, one must take into account its effects on modulatory presynaptic/prejunctional nicotinic receptors, which are located on different axon terminals containing transmitters. Several subtypes of nAChRs have been distinguished by ligand binding studies, (cf. refs. 23 and 28) protein chemistry,[29] and molecular biological techniques (cf. refs. 3 and 25). The disadvantage of these techniques is that the ligands used until now lacked sufficient selectivity and could not be used for precise anatomical localization of the nAChRs, in particular, to distinguish between pre- and postsynaptic sites and the types of neurons on which these sites are localized. Because *in situ* hybridization studies suggest that more than one variant of α and β subunit combinations exist in the CNS, the study of a broad concentration range of different agonists and antagonists for their ability to release NE and inhibit NE release, respectively, from the hippocampus enabled us to classify the subtype of presynaptic nAChRs.

Presynaptic nAChRs

We examined NE release in the hippocampus. Several studies provided evidence that a high concentration of (−)nicotine ($> 100\ \mu M$) stimulates transmitter release in a $[Ca^{2+}]_o$-independent manner and that the release cannot be blocked by classical nAChR antagonists.[30,31] Many other studies, in contrast, have shown that (−)nicotine is able to release transmitter in a $[Ca^{2+}]_o$-dependent manner.[12,20,31–34] It has been reported[23] that at least three different subtypes of nAChR may exist in the hippocampus, possibly with unique functional characteristics. Although the $\alpha 7$ subunit appears to be the predominant subtype on the basis of *in situ* hybridization studies in the hippocampus[35] and electrophysiological responses,[36] $\alpha 2$–5 and $\beta 2$ subunits have also been demonstrated. To classify the subtype of nAChR involved in the $[Ca^{2+}]_o$-dependent release of NE from the hippocampus, the effect of different nAChR agonists and antagonists was studied.

After the tissues had been loaded with [^{3}H]NE, the spontaneous and stimulation-evoked release of [^{3}H] NE was measured (FIG. 1), and the effect of different agonists

TABLE 2. Norepinephrine Releasing Effect of Dimethylphenyl Piperazinium Iodide (DMPP) in Isolated CA1, CA3, and Dentate Gyrus Subregion of Hippocampus

	Fractional Release at $Rest_2$/Fractional Release at $Rest_1$			
	CA1	CA3	DG	Hippocampus (CA1 + CA3 + DG)
DMPP				
1 μM	0.91 (1)[a]	0.91 (1)	1.12 (1)	1.14 ± 0.03[b] (4)
3 μM	1.26 ± 0.07[b] (3)	1.02 ± 0.07 (3)	1.42 ± 0.13[b] (3)	
10 μM	1.67 ± 0.12[b] (5)	1.30 ± 0.09[b] (5)	1.73 ± 0.12[b] (5)	1.85 ± 0.04[b] (4)
20 μM	1.66 ± 0.12[b] (10)	1.60 ± 0.15[b] (8)	2.02 ± 0.14[b] (8)	2.23 ± 0.09[b] (4)
50 μM	1.66 ± 0.09[b] (4)	1.58 ± 0.09[b] (4)	2.11 ± 0.26[b] (4)	
100 μM	1.66 ± 0.14[b] (3)	1.59 ± 0.09[b] (4)	2.33 ± 0.31[b] (3)	4.41 ± 0.10[b] (4)
Control	0.94 ± 0.04 (5)	0.90 ± 0.02 (5)	0.92 ± 0.04 (6)	0.91 ± 0.02 (4)

NOTE: Male Wistar rats, weighing 150–160 g, were killed by a blow to the head under light ether anesthesia. The brain was quickly removed and the hippocampus dissected on ice and sliced into 0.4 mm thick coronal sections with a McIlwain chopper. Slices were dissected into tissue parts containing mainly CA1, CA3, and dentate gyrus. The tissues were incubated at 37 °C for 40 min in Krebs solution (in mM: NaCl, 113; KCl, 4.7; $CaCl_2$, 2.5; KH_2PO_4, 1.2; Mg_2SO_4, 1.2; $NaHCO_3$, 25.0; and glucose, 11.5) containing 1-[7,8-^{3}H]norepinephrine (490 KBq/mL, 555 GBq/mmol spec. act., Amersham). Krebs solution contained ascorbic acid (3×10^{-3} M), Na_2EDTA (10^{-4} M). Release of tritium was expressed in Bq/g and as percentage of the radioactivity (fractional release, FR) in the tissue at the time the sample was collected. DMPP was added to the Krebs solution in the 8th fraction and kept throughout the experiments. FRR_2/FRR_1 was calculated.

[a]Number of experiments is in parentheses.

[b]Significance at the level of $p < 0.05$. Mean ± SEM.

No significant difference was found in the effect of DMPP in the three subregions of the hippocampus. In addition various nAChR agonists were tested for their ability to release NE from hippocampal slice preparations. (+)Anatoxin-a is an important probe for characterizing nAChRs[37] and has much greater affinity for high-affinity (−)nicotine sites (K_i = 0.34–3.5 nM)[34] than for muscarinic binding sites. It inhibits muscle twitch in rat[38] and at 1 μM concentration blocks the indirect twitch of the frog sciatic nerve-sartorius preparation in a short time.[39] This effect is associated with its AChR ion channel-blocking action exerted at higher concentration.[40] At nanomolar concentrations it is a selective agonist.[41] In our experiments it was also effective in releasing NE from hippocampal slices. The rank order of potency of the nAChR agonist for releasing NE from the hippocampal slice preparation was (+)anatoxin-a > DMPP > (−)nicotine ≫ cytisine, with the equipotent (ED_{20} of DMPP) values 1.2, 2.3, 10.2, and 22.0 μM, respectively. DMPP, unlike other agonists, enhanced the stimulation-evoked release, but in contrast, this effect was not antagonizable with nAChR antagonists. To rule out the possibility that the effect of nAChR agonists is indirect, mediated through glutamic acid release,[40] experiments were performed in the presence of MK-801 (3 μM). Under these conditions DMPP enhanced the release of NE to the same extent. Thus, it is unlikely that glutamic acid released by nAChR stimulation is responsible for the release of NE.

Various nAChR antagonists were tested for their ability to prevent the release of NE induced by 20 μM DMPP, a concentration of DMPP that gives near half-maximal release (see TABLE 2). Mecamylamine and other antagonists [(+)tubocurarine and hexamethonium] prevented the increase by DMPP of NE release. α-Bungarotoxin (αBGT), a potent nAChR blocker at the neuromuscular junction, applied at a concentration of 3 μM for 10 min, and dihydro-β-erythroidine (DHβE) (10 μM)

did not affect the response to DMPP (TABLE 3). The rank order of potency was mecamylamine > (+)tubocurarine > hexamethonium ≫ αBGT = DHβE.

$[Ca^{2+}]_o$-Dependence of nAChR-mediated Release of Norepinephrine

When $[Ca^{2+}]_o$ was omitted and EGTA (1 mM) added to the Krebs solution, DMPP (20 μM) had no effect on [^{3}H]NE release from the CA1 region: FRR2/FRR1 = 0.97 ± 0.11 (n = 4) measured in the presence of DMPP was not different from control (0.94 ± 0.04, $p > 0.1$). The nAChR is a ligand-operated ion channel whose activation results in membrane depolarization and Ca^{2+} influx and subsequent release of different transmitters. It has been suggested that Ca^{2+} influx through nAChRs accounts for some effects of nAChR agonists in the nervous system[33,42,43] and that this is a distinctive property of neuronal receptors in contrast to muscle nicotinic receptors.[43] The findings that the effect of DMPP was $[Ca^{2+}]_o$-dependent, that Cd^{2+} (100 μM) partly prevented the effect of DMPP (data not shown), and that nifedipine, even at a high concentration, had no effect indicate that the stimulation of nAChRs opens N-type Ca^{2+} channels.

Atropine, a muscarinic antagonist (1 μM) that totally blocks muscarinic responses in other systems, and oxotremorine (1 μM), a muscarinic agonist, were without effect on the release of NE.[44] This makes the noradrenergic pathway different from others, where noradrenergic axon terminals express inhibitory muscarinic receptors. The presence of nAChRs means that a possibility exists for excitatory input by cholinergic neurons.

The release of [^{3}H]NE from rat vas deferens[20] was also measured. nAChR agonists released NE with the following rank order of potency: (+)anatoxin-a > DMPP > (−)nicotine > cytisine; ED_{20} = 0.8 > 5.1 > 10.2 > 52.3 μM (ED_{20} indicates that the release was enhanced by 20%).

TABLE 3. Apparent Dissociation Constants (K_d) of Different nAChR Antagonists at Presynaptic nAChRs of Noradrenergic Axon Terminals in the Hippocampus of the Rat

Antagonist	Dissociation Constant (μM)
Mecamylamine	1.05 ± 0.05 (6)
(+)Tubocurarine	17.65 ± 1.40 (5)
Hexamethonium	35.20 ± 2.15 (4)
Pancuronium	21.21 ± 2.00 (5)
α-Bungarotoxin	≫30 (5)
Dihydro-β-erythroidine	≫30 (3)

NOTE: In hippocampal slice preparations the apparent equilibrium dissociation constant (K_d) for nAChR antagonists was determined by the dose-ratio method. DMPP was used as agonist. The following equation was used to relate the dissociation constant (K_d) to the dose-ratio and the antagonist concentration $K_d = a/DR-1$, where DR is the concentration-ratio, i.e., the EC_{50} value for agonist in the presence of the antagonist divided by the ED_{50} value in the absence of antagonist and a is the concentration of antagonist. EC_{50} indicates the concentration of agonist needed to produce a 50% increase of R_2/R_1 value. Three to six different concentrations of agonists were used to establish a concentration-response curve. Mean ± SEM. Number of experiments is in parentheses.

Modulation by α_2-Adrenoceptors of nAChR-mediated Release of Norepinephrine

It is known that the action potential-evoked release of NE is subject to α_2-adrenoceptor-mediated inhibition; therefore, the question arises whether the nAChR-mediated release, which is $[Ca^{2+}]_o$-dependent, but not associated with axonal firing, can be modulated. Neurochemical evidence was obtained that xylazine, an α_2-adrenoceptor agonist, prevented DMPP from releasing NE. 7,8(Methylenedioxy)-14α-hydroxyalloberbane (CH-38083), a selective α_2-adrenoceptor antagonist,[45] antagonized the effect of xylazine, indicating that this action was mediated via α_2-adrenoceptors (TABLE 4). The effect of α_2-adrenoceptor stimulation might have been mediated by decrease of presynaptic Ca^{2+} channels[46] and/or by increase of K^+ conductance of the presynaptic axon terminal. Evidence has been obtained that the

TABLE 4. Inhibition by α_2-Adrenoceptor Stimulation of [^{3}H]Norepinephrine Release Induced by DMPP in Rat Hippocampal Slices

Treatment	FRR_2/FRR_1	Significance p
1. Control	0.95 ± 0.03	
2. Xylazine, 3 μM	0.98 ± 0.10	
3. CH-38083, 1 μM	0.94 ± 0.06	
4. DMPP, 20 μM	2.23 ± 0.09	4:1 < 0.001
		5:1 > 0.05
5. Xylazine, 3 μM + DMPP, 20 μM	1.19 ± 0.06	
6. CH-38083, 1 μM + DMPP, 20 μM	2.21 ± 0.17	6:1 < 0.001
		6:4 > 0.05
		6:5 < 0.01
7. CH-38083, 1 μM + xylazine, 3 μM	1.92 ± 0.20	7:1 < 0.001
+ DMPP, 20 μM		7:4 > 0.05
		7:5 < 0.01
		7:6 > 0.05

NOTE: CH-38083, 7,8(methylendioxy)-14α-hydroxyalloberbane, a selective α_2-adrenoceptor antagonist,[45] was added to the perfusion fluid 6 min prior to administration of xylazine, an α_2-adrenoceptor agonist. Fractional release at rest, FRR_1, was estimated at the third and fourth fractions, and FRR_2 at the tenth and eleventh fractions. Drugs were added between the two, 10 min prior to R_2 and kept throughout the experiment. Krebs solution, 37 °C. Significance, one-way analysis of variance followed by a Tukey-Kramer multiple comparisons test. DMPP, dimethylphenyl piperazinium iodide.

stimulation of α_2-adrenoceptors reduces the activity of N-type Ca^{2+} channels,[46] the dominant calcium entry pathway triggering sympathetic transmitter release.[47] It is conceivable that the effect of nAChR is mediated via stimulation of N-type Ca^{2+} channel located on axon terminals.

In contrast to the nAChR located on the striated muscle, which is assembled from four different subunits arranged as the pentamer $\alpha 2\beta\gamma\delta$, several findings suggest that the neuronal receptor is composed of α and β subunits only.[29]

The finding that the rank order of antagonists was mecamylamine > (+)tubocurarine > hexamethonium and αBGT or DHβE were completely ineffective indicates that the presynaptic nAChRs involved in NE release do not contain the α7 subunit, one of the predominant subtypes of nAChRs in the hippocampus and one involved in long-term potentiation.[36] Binding studies suggest that cytisine is a more potent

TABLE 5. Rank Order of Potency of Different nAChR Agonists and Antagonists and the Possible Subunit Composition of nAChRs

Subunits		Rank Order of Potency of	
		Agonist	Antagonist
	Neuronal nAChRs		
	Presynaptic		
	NE release		
α3β2	Hippocampus (1)	(+)ATX-a > DMPP > (−)Nic ≫ Cyt	Mec > dTC > Hex ≫ α-BGT = DHβE
	Vas deferens (2)	(+)ATX-a > DMPP > (−)Nic > Cyt	
	DA release		
α3β2	Striatum (3)	ACh > (−)Nic = Cyt > DMPP > Anab > Lob	n-BGT > Mec > DHβE > Hex > dTC ≫ α-BGT
	(4)	Cyt = (−)Nic = DMPP	Neosurtx > DHβE > Mec ≫ α-BGT
	ACh release		
	Neuromuscular junction (5)		dTC > Panc > Hex ≫ α-BGT
	(6)	(−)Nic = Cyt	
	Postsynaptic		
α4β2	Somatodendritic		
	Myenteric plexus (7)	(+)ATX-a > DMPP = (−)Nic > Cyt	DHβE = Mec > dTC > Hex ≫ α-BGT
	Substantia nigra (8)	(−)Nic	DHβE = Mec
	Muscle nAChRs		
α2β1εδ	Striated muscle (9)		Panc > dTC > α-BGT ≫ Hex = Mec

Abbreviation: NE, norepinephrine; DA, dopamine; ACh, acetylcholine; (+)ATX-a, (+)anatoxin-a; DMPP, 1,1-dimethyl-4-phenylpiperazinium iodide; (−)Nic, (−)Nicotine; Anab, (±)Anabasine; Lob, (−)lobeline HCl; Hex, hexamethonium HBr; dTC, (+)tubocurarine; α-BGT, α-bungarotoxin; Mec, mecamylamine; DHβE, dihydro-β-erythroïdine, Cyt, cytisine; Panc, pancuronium; Neosurtx, neosurugatoxin.

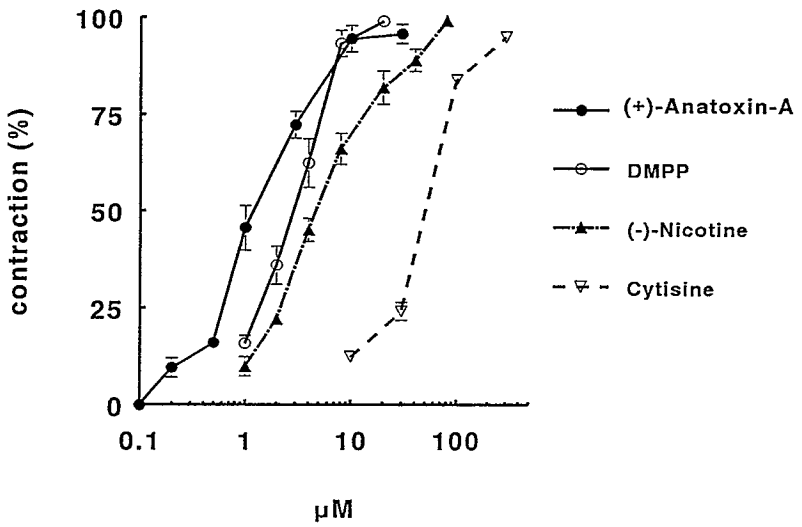

FIGURE 2. Effect of nAChR agonists on isolated longitudinal muscle strip with myenteric plexus attached (guinea pig ileum). For method see reference 49.

agonist than (−)nicotine.[31,48] However, its potency depends on the subunit composition of the nAChR. *In situ* hybridization has revealed the existence of α- and β-subunits in different regions of the brain, including the hippocampus.[26,27] Cytisine can distinguish between receptors containing β4, which are highly sensitive to cytisine, and receptors containing β2, which are completely insensitive. Therefore it is suggested that the presynaptic nAChRs, whose stimulation by nAChR agonists [(+)anatoxin-a > DMPP > (−)nicotine ≫ cytisine] resulted in an increase of NE in the three different regions of the hippocampus, contain the β2 subunit (TABLE 5). αBGT-insensitive nAChRs, which represent a large population of nAChRs present throughout the hippocampus, are most likely composed of a combination of α3 and β2 subunits, and are involved in the release of NE by nAChR stimulation.

In the striatum the dopaminergic axon terminals are equipped with similar nAChRs.[31] It is very likely that these nAChRs are composed of α3 and β2 subunits. Although n-bungarotoxin and neurosuguratoxin were very potent in inhibiting DA release evoked by nAChR stimulation, αBGT had no effect at all.[12,31] Because the potency of nAChR agonists depends on the α-β combination,[24] the rank order of agonist potency for DA release obtained by Grady *et al.*[31] suggests an α3β4 combination (relatively high cytisine activity), although it is also not a perfect match. However, inasmuch as nBTX is not active on nAChRs composed of α3β4 subunits, the suggestion of Grady *et al.*[31] seems very unlikely, and therefore the nAChRs responsible for DA release are most likely composed of α3β2 subunits.

There is convincing neurochemical evidence that at the mouse neuromuscular junction (phrenic nerve–hemidiaphragm preparation) there are nAChRs presynaptically involved in positive feedback modulation of ACh release.[18] Presynaptically the rank order of potency of nAChR antagonists was (+)tubocurarine > pancuronium > hexamethonium, and αBGT had no effect. In contrast, postsynaptically αBGT was very active (TABLE 5), and hexamethonium had no effect. This, in fact, is the first

evidence that in a cholinergic synapse the pre- and postsynaptically located nAChRs are heterogeneous and their subunit composition is different.

Somatodendritic nAChRs

The nAChR agonists produced increasing contractions of the longitudinal muscle of guinea-pig ileum[49,50] (FIG. 2), an effect certainly due to the release of ACh from cholinergic interneurons of the Auerbach plexus,[50–53] because the smooth muscle is not equipped with nicotinic receptors, and the somatodendritic part of cholinergic interneurons is equipped with nAChRs. In fact, nAChR agonists stimulate nAChRs located on the somatodendritic part of the neurons[50,51] subsequently producing firing

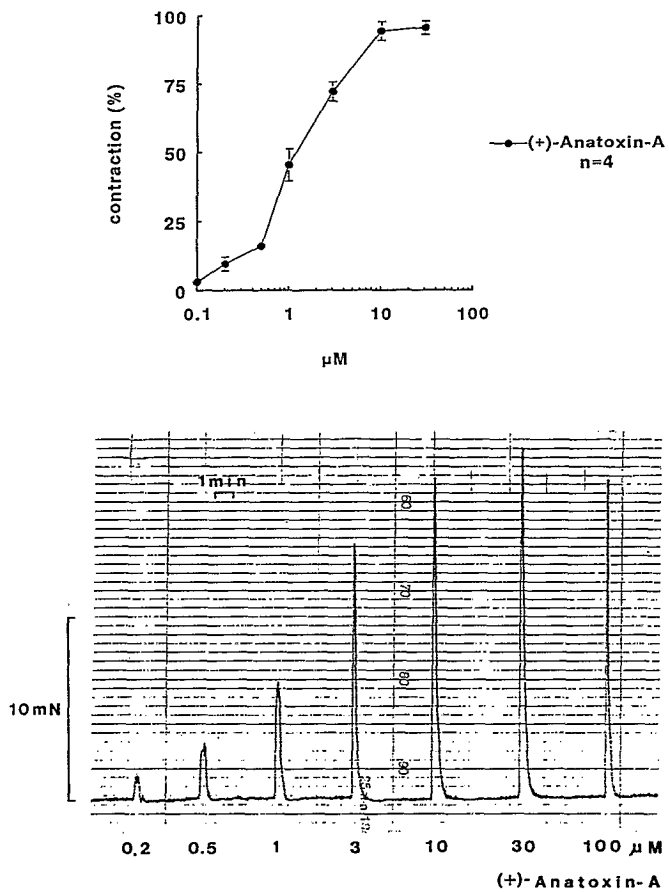

FIGURE 3. Effect of (+)anatoxin-A on isolated longitudinal muscle strip preparation. For method see reference 49.

of the neurons and resulting in release of ACh.[51,53] First the concentration-response curve for the specific nicotinic agonist DMPP was determined. At 100 μM concentration, DMPP produced the maximum contraction (ED_{50} = 2.96 ± 0.34 μM), then decreased responses at higher concentrations. (−)Epibatidine, (+)anatoxin-a, (−)nicotine, and cytisine also produced contractions in a concentration-dependent manner. (−)Epibatidine is an alkaloid isolated from the skin of the poison frog, *Epipedobates tricolor.*[54] A nAChR agonist,[55] it inhibits [^{3}H]cytisine binding with an

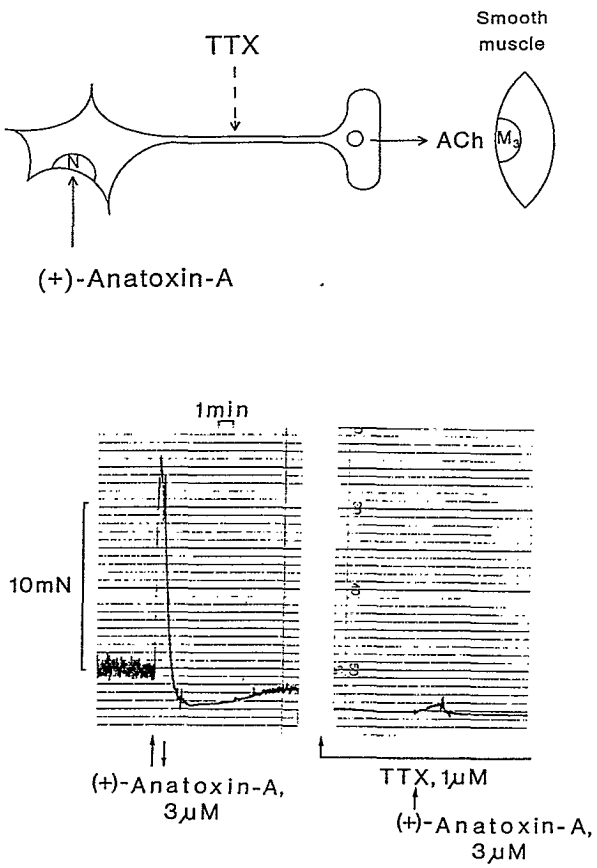

FIGURE 4. Evidence that the site of effect of (+)anatoxin-A is on the somatodendritic part of the cholinergic interneurons. Note that tetrodotoxin (TTX) inhibited the effect of (+)anatoxin-A to produce contraction. TTX blocks nerve conduction; therefore, the stimulation of somatodendritic nAChRs does not produce firing and subsequent ACh release and M_3 muscarinic subtype-mediated contraction of the smooth muscle.

IC_{50} of 70 pM and with a K_i of 43 pM. In our experiments the ED_{50} was 0.03 ± 0.01 μM. Its effect was readily antagonized by mecamylamine (K_d = 0.8 μM). The contraction was very fast and showed no tendency to build up desensitization. Atropine and TTX blocked its effect to produce contraction, indicating that its action is mediated via ACh released in response to somatodendritic stimulation of cholinergic interneurons. Maximum responses occurred at a concentration of 10 μM of (+)anatoxin-a (FIG. 3). Peak contraction developed quickly at concentrations above

TABLE 6. Effect of Different nAChR Agonists on Somatodendritic Nicotinic Receptors Located on Cholinergic Interneurons

Agonists	EC_{50} (μM)	*N*
(−)Epibatidine[a]	0.03 ± 0.01	6
(+)Anatoxin-a	1.38 ± 0.23	4
DMPP[b]	2.96 ± 0.34	11
(−)Nicotine	5.87 ± 0.24	14
Cytisine	50.00 ± 2.00	3

NOTE: To study the effect of nAChR agonists and antagonists on somatodendritic nAChRs, 4–5 cm long, longitudinal muscle strips of guinea-pig ileum[49] were suspended in Krebs solution in a 2-mL organ bath. A resting tension of 10 mN was applied to the muscle. The dose-response of nAChR agonists was determined by adding successively higher concentrations to the bath. From the computer-derived, best-fit, log dose-response regression line, the concentration of nAChR agonist that increased the isometric force of muscle contraction to 50% of the maximum (EC_{50}) was calculated.

[a]Exo-2(6-chloro-3-pyridyl)-7-azabicyclo heptane.[54,55]

[b]1,1-Dimethyl-4-phenylpiperazinium iodide.

5 μM but decreased before washout, that is, the contraction was not maintained. This decrease represented either receptor desensitization or a block induced by the agonist applied. When the preparation was exposed to ACh (0.05–3 μM) the contraction was maintained. Atropine completely blocked the responses, indicating that the response of the smooth muscle is mediated by ACh released from varicosities via stimulation of muscarinic receptors located on the smooth muscle cells. In addition, TTX, a sodium channel blocker, completely abolished smooth muscle contractions (FIG. 4), and the release of ACh evoked by nAChR agonists such as DMPP, (−)nicotine,[51] or (+)anatoxin-a,[52] and rather selective nAChR antagonists antagonized the effects of these agonists. Therefore, it is suggested that this method is useful for studying the potency of different nAChR agonists and antagonists,

TABLE 7. Apparent Dissociation Constant (K_d) of Different nAChR Antagonists Estimated on Somatodendritic Nicotinic Receptors

	K_d (μM)	
Antagonists/Agonists	DMPP	(−)Nicotine
Dihydro-β-erythroidine	0.63 ± 0.08 (4)	
Mecamylamine	0.95 ± 0.28 (7)	1.26 ± 0.26 (4)
(+)Tubocurarine	2.87 ± 0.61 (6)	1.36 ± 0.41 (8)
Hexamethonium	10.37 ± 2.79 (10)	14.41 ± 3.46 (6)
α-Bungarotoxin	>20	

NOTE: To estimate the apparent dissociation constant of different antagonists versus different agonists the following equation was used: a/DR-1. The apparent dissociation constant (K_d) of antagonists, a concentration required to double the ED_{50} of agonists for the increase of the force of muscle contraction by nAChR agonists, was calculated. The negative logarithm of this concentration, pA_2 is commonly accepted as a measure of antagonist affinity. The maximal contractile response to nAChR agonists was designated as 100%, and the ED_{50} of agonists were calculated. The effect of oxotremorine, a selective muscarine receptor agonist that induced contraction of the muscles, was also investigated in the presence of different concentrations of nAChR antagonists studied. The nAChR antagonists did not affect oxotremorine on the smooth muscle, indicating that they do not exert antimuscarinic activity. Number of experiments is in parentheses.

provided they do not possess antimuscarinic activity at the concentration applied. When the equipotent concentration (EC_{50}) of different agonists was calculated (TABLE 6), (−)epibatidine, a nAChR agonist with strong analgesic activity, proved to the most potent agonist and cytisine the least potent. However, when the apparent dissociation constants (K_d) of antagonists were estimated (TABLE 7) DHβE was seen to be the most potent, and the rank order of potency was DHβE > mecamylamine > (+)-tubocurarine > hexamethonium ≫ αBGT. The rank order of potency of agonists for somatodendritic receptors [(+)anatoxin-a > DMPP = (−)nicotine > cytisine] differs from that found in the hippocampus. The finding that cytisine is less potent than DMPP and (−)nicotine (EC_{50} = 47.5 ± 2.0 μM) indicates that the β2 subunit is involved in forming somatodendritic nAChRs. In addition, that DHβE was very active and αBGT had no effect suggests that the α4-subunit is also present. Flores *et al.*[56] suggested that α4 and β2 subunits are associated in forming the predominant, and possibly the only, subtype of neuronal nicotinic receptor with high affinity for agonists. They found that all α4-subunits that were labeled by [^{3}H]cytisine were coupled to β2 subunits. In addition it was shown[57] that neuronal α4β2 nAChRs are insensitive to αBGT, but they are sensitive to blockade by DHβE.[48] Taking into account our data, it is suggested that on the postsynaptic site in the neuron-neuron synapse the nAChRs are composed of α4β2 subunits.

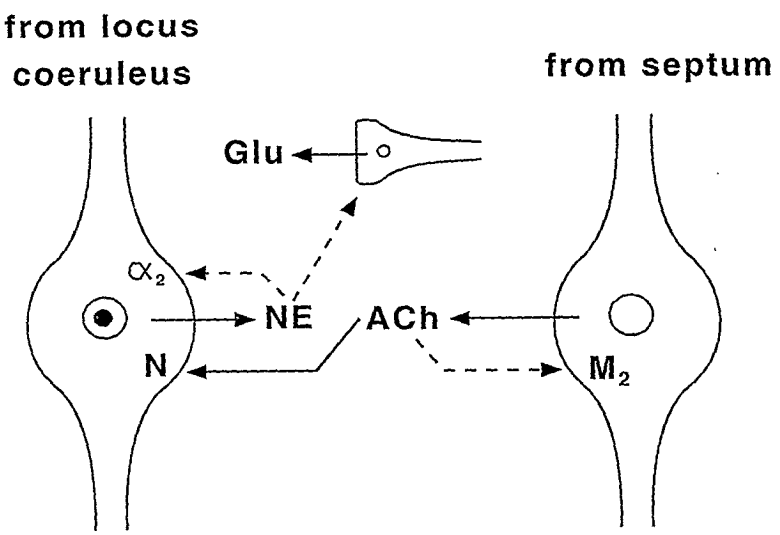

FIGURE 5. Interaction between cholinergic input and noradrenergic axon terminals in the hippocampus. ACh released from the cholinergic terminals may be able to stimulate nAChRs located on the noradrenergic terminals and release norepinephrine (NE). Note that the noradrenergic axon terminals are not equipped with inhibitory muscarinic receptors;[44] thus the effect of ACh on nAChRs is unopposed.

TABLE 8. Characteristics of nAChR Stimulation-mediated Release of Norepinephrine. Different Types of Transmitter Release

	Associated with AP[a]	$[Ca^{2+}]_o$-Dependent	Subject to Presynaptic Modulation	Carrier-mediated
Resting	Not	Not	Not	Yes (partly)
Electrical stimulation	Yes	Yes	Yes	Not
nAChR-mediated	Not	Yes	Yes	Not
Reversal of the carrier	Not	Not	Not	Yes

[a]AP, action potential.

Possible Role of Presynaptic nAChR Located on the Noradrenergic Axon Terminals in the Hippocampus

Inasmuch as the hippocampus receives noradrenergic innervation from the locus coeruleus and NE inhibits excitatory glutamate-mediated synaptic transmission in area CA3,[37,58,59] the nAChR-mediated NE-releasing action of cholinergic innervation and the α_2-adrenoceptor-mediated inhibition of this interaction described in this paper may have a functional role. The effectiveness of this interaction is multiplied by the fact that the noradrenergic axon terminals are not subjected to muscarinic receptor-mediated inhibition (FIG. 5). It means that the firing of cholinergic afferents and the subsequent release of ACh may produce an increase of NE release that is not associated with axonal firing. The release of NE in response to activation of cholinergic input in the hippocampus may result in a decrease of the evoked release of transmitter from excitatory terminals of both mossy fibers and CA3 pyramidal cell recurrent collaterals;[59] thus the stimulation of nAChRs located on the noradrenergic axon terminals would result in reduction of the activity of pyramidal cells.

It is generally accepted that both the cholinergic system and the hippocampus play a significant role in memory and learning. Recent data, however, suggest that the interaction of cholinergic and noradrenergic pathways may be even more important for memory processes.[60] Several studies concerning presynaptic inhibition of transmitter release from the hippocampus have shown, using electrophysiological[cf.61] and neurochemical[42,62] methods, that the release of different transmitters can be modulated via stimulation of presynaptic receptors. The recent finding of excitatory interaction between cholinergic input and noradrenergic neurons through nAChRs located on noradrenergic axon terminals, and of the absence of inhibitory muscarinic receptors on the noradrenergic axon terminals[44]—in contrast to the axon terminals with opposite muscarinic and nicotinic actions on the same population of axon terminals (e.g., the nigrostriatal pathway)—make this interaction very effective.

In summary, the stimulation of nAChRs located on the axon terminals results in a release of NE and other transmitters, but unlike that observed at rest, it is $[Ca^{2+}]_o$-dependent and is subject to presynaptic modulation (TABLE 8).

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